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Cadmium activates CaMK-II and initiates CaMK-II-dependent apoptosis in mesangial cells

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Abstract **Cadmium is a toxic metal that initiates both mitogenic responses and cell death. We show that Cd^{2+} increases phosphorylation and activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II) in mesangial cells, in a concentration-dependent manner. Activation is biphasic with peaks at 1–5 min and 4–6 h. Cadmium also activates Erk, but this appears to be independent of CaMK-II. At 10–20 μM, Cd^{2+} initiates apoptosis in 25–55% of mesangial cells by 6 h. Inhibition of CaMK-II, but not of Erk, suppresses Cd^{2+}-induced apoptosis. We conclude that activation of CaMK-II by Cd^{2+} contributes to apoptotic cell death, independent of Erk activation.**

Keywords: Cadmium toxicity; CaMK activation; Apoptosis; Mesangial cells; MAP kinase

1. Introduction

Cadmium is an environmentally and occupationally important toxic metal with a long biological half-life. It acts as a cancer promoter through mitogenic effects on gene expression (reviewed in [1]) and produces malignant tumors in experimental animals [2–5]. Cadmium is both pro- and anti-apoptotic in circumstances that probably depend on both cell type and duration and conditions of treatment. Thus it has been shown to induce apoptosis in T lymphocytes [6], LLC-PK_1 cells [7], canine proximal tubules [8], and rat testicular tissue [9]. It induces apoptosis by both caspase-dependent [10–12] and caspase-independent [13] pathways. However, Cd^{2+} has also been reported to block apoptosis induced by a variety of agents, e.g., in CHO cells where the effect was attributed to the inhibition of caspase-3 [14]. We recently demonstrated inhibition by Cd^{2+} of both intrinsic and extrinsic apoptotic pathways in mesangial cells following initiation by camptothecin and TNF-α, respectively [15].

Three major mitogen-activated protein kinase (MAPK) pathways occur in mammalian cells, involving Erk, Jun kinase (Jnk) and p38 [16]. In general, Erk is activated by growth factor receptors and stimulates cell proliferation, whereas Jnk and p38 are responsive to genotoxic agents and stresses [17–19]. Cadmium is a known regulator of each pathway [19–21], albeit with differential effects in different cell types. All three MAPK pathways are activated by Cd^{2+} in mesangial cells ([22,23] and data not shown). In addition to the MAPKs, Ca^{2+}/calmodulin-dependent kinase II (CaMK-II) is a general integrator of Ca^{2+} signaling that has been linked to oncogene induction in several cell lines [24,25]. The isoform CaMK-II is present in mesangial cells [26,27]. Cadmium activates CaMK in cell-free systems [28,29], although we previously reported that short-term treatment of rat mesangial cells with Cd^{2+} did not activate CaMK-II in these cell extracts [30].

CaMK-II has been linked to regulation of apoptosis. Hepatocytes undergoing apoptosis following inhibition of protein phosphatases were protected from DNA fragmentation and polarized budding when CaMK-II inhibitors were added up to 20 min after phosphatase inhibition [31], indicating a CaMK-II-dependent commitment step at this time. The related kinases, CaMK-IV and CaMK-like kinase are both cleaved by caspase-3 to facilitate neuronal apoptosis [32,33], although CaMK is not consistently pro-apoptotic. Thus, CaMK-IV inhibits apoptosis in neurons deprived of K^+ [34]. Furthermore, CaMK-II maintains c-FLIP phosphorylation, rendering apoptosis-resistant glioma cells proliferative in response to Fas agonist, and over-expression of CaMK-II makes sensitive cells resistant [35].

In the present study, we demonstrate that Cd^{2+} activates CaMK-II in mouse mesangial cells and initiates both apoptotic and necrotic cell death. Inhibition of CaMK-II increases cell viability in the presence of Cd^{2+}, primarily due to suppression of apoptosis.

2. Materials and methods

2.1. Cell culture

Mesangial cells were isolated from kidneys of 129/Sv mice using Dynabeads as described [36], and characterized as homogeneous cultures by morphology and staining for α-smooth muscle actin. Cells were cultured in RPMI 1640 medium with 10% FBS and used between passages 5 and 20. Before exposure to $CdCl_2$, cells were rendered quiescent at 70–80% confluence by growth in medium containing 0.2% FBS for 48 h. Quiescent cells were then washed with PBS and pretreated with inhibitors in serum-free medium for 1 h before the addition of $CdCl_2$ for up to 6 h. The inhibitors used (all from CalBiochem, San Diego, CA) were KN93 (an inhibitor of the calmodulin binding/activation site) and its inactive analogue KN92, KT5926 and K252a (competitive inhibitors of ATP binding at the catalytic site), and myristoylated autocamtide-2-related inhibitory peptide (AIP, a peptide analogue of the autoinhibitory domain of CaMK-II) at concentrations of KN93/KN92 = 10 μM, KT5926 = 100 nM,

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Abbreviations: CaMK, Ca^{2+}/calmodulin-dependent kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide

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K252a = 40 nM, and AIP = 100 μM. AIP was introduced into the MMC by reversible permeabilization, as described previously [37]. Briefly, starved cells were slowly cooled to 4 °C by sequential 2-min incubations with PBS. The cells were then incubated with freshly prepared permeabilization buffer (20 mM HEPES, pH 7.4, with 10 mM EGTA, 140 mM KCl, 50 μg/mL saponin, 5 mM NaN_3, 6 mM ATP, and 5 mM oxalic acid dipotassium salt) containing 100 μM myristoylated-AIP for 20 min on ice. The cells were then gently washed four times with chilled PBS. After a 20-min recovery on ice in PBS, the cells were placed at room temperature for 2 min and then incubated in serum-free medium at 37 °C for 30 min before treatment with $CdCl_2$.

2.2. Western blotting

Cells were lysed by two freeze–thaw cycles in 50 mM HEPES buffer containing 0.5% Nonidet P-40, 1 mM Na_3VO_4, 25 mM NaF, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin. The mixture was then sonicated twice for 5 s at 200 W and centrifuged (15 000 × *g*, 15 min). Cell extracts were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS containing 0.1% Tween 20 and blotted overnight at 4 °C with anti-phospho-Thr_{286}-CaMK-II (1:2000; BioSource, Camarillo, CA), anti-phospho-Erk (1:2000; Cell Signaling, Beverly, MA), anti-Erk-2 cross-reactive with both p42 and p44 Erk (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α-smooth muscle actin (1:5000, Sigma, St. Louis, MO). Anti-CaMK-II-δ2 was generously provided by Harold Singer [38]. Signals were developed with horseradish peroxidase-conjugated secondary Ab at room temperature for 60 min. Blots were washed three times with TBS/Tween 20 and visualized with ECL Plus reagent (Amersham, Baie d'Urfé, PQ) according to the manufacturer's instructions.

2.3. CaMK-II activity

Autonomous CaMK activity and total activity were measured as described previously [27]. For autonomous activity, 5–10 μg of protein were incubated at 30 °C for 3 min in 25 μl of 50 mM HEPES assay buffer containing 10 mM $MgCl_2$, 0.1 mM ATP, 1 mM EGTA, 0.02 mM autocamtide peptide (KKALRRQETVDAL) as substrate, and 5 μCi/ml [γ-^{32}P] ATP. For total activity, EGTA was replaced by 4 mM $CaCl_2$ and 2 μM calmodulin. Reaction was stopped by addition of ice-cold TCA to 5%. The reaction mixture was spotted on P81 phosphocellulose filters, washed with 75 mM H_3PO_4, and counted by liquid scintillation.

2.4. Cell viability assay with thiazolyl blue (MTT)

Cells growing in 24-well plates were exposed to 1 mg/ml MTT in 200 μl phenol red-free RPMI 1640 for 1 h at 37 °C. The medium was removed, and 500 μl of DMSO was added with shaking for 30 min at room temperature. Samples (100 μl) were placed in a 96-well plate, and the absorbance read at 570 nm with background subtraction at 650 nm.

2.5. Detection of cell death by flow cytometry

To detect phosphatidylserine externalization, 1×10^5 cells were suspended in 100 μl binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 μl Annexin V-FITC (BD Bioscience, Mississauga, ON) and 5 μg/ml propidium iodide (PI; Sigma), and incubated for 15 min at room temperature. Then 400 μl binding buffer was added and the samples were analyzed immediately in an Epics Elite flow cytometer (Beckman Coulter). Live cells were considered to be Annexin V −ve/PI −ve; apoptotic cells Annexin V +ve/PI −ve, late apoptosis/necrosis Annexin V +ve/PI +ve.

2.6. Statistical analyses

Values are expressed as means ± S.D. and differences are evaluated by ANOVA with post hoc testing by Dunnett's test against control (Table 1, Fig. 1C) or Tukey's test for multiple within-group comparisons (Table 2).

Table 1
Cell viability by the MTT assay

Conditions	Viability (%)	Difference vs. Cd alone
Serum-free	79.5 ± 4.5	$P < 0.001$
Cd	28.7 ± 8.7	—
Cd + PD98059	27.6 ± 13.0	N.S.
Cd + KN93	45.0 ± 6.2	$P < 0.05$

Quiescent mouse mesangial cells were transferred to serum-free medium with or without 15 μM $CdCl_2$ for 6 h. The Erk inhibitor PD98059 (50 μM) or the CaMK-II inhibitor KN93 (10 μM) were included in some cultures. Values are mean ± S.D. from six experiments and are expressed as a percentage of the MTT absorbance in quiescent cultures prior to transfer to serum-free conditions. All Cd^{2+} treatment groups differed from serum-free alone at $P < 0.001$.

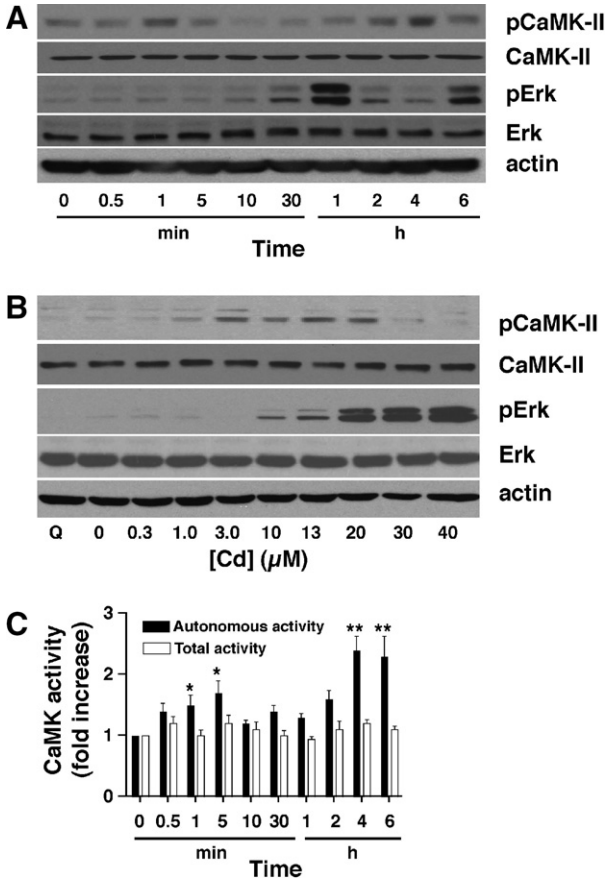

Fig. 1. Activation of CaMK-II and Erk by Cd^{2+} in mesangial cells. (A) Western blots of CaMK-II phosphorylated on Thr_{286} (pCaMK) and phosphorylated p42/p44 Erk (pErk) after treatment of mouse mesangial cells with 10 μM $CdCl_2$ for the times indicated. The blots are typical of at least three independent experiments in each case. β-Actin is immunoblotted on the same gels to serve as a loading control, and total Erk and CaMK-II are also shown as evidence that gene expression is not influenced. (B) Western blots of pCaMK and pErk (as in panel A) in cell extracts from mesangial cells exposed for 6 h to the indicated concentrations of Cd^{2+} as $CdCl_2$. The blot is typical of at least three experiments at most concentrations. (C) Measured CaMK activity (total and autonomous using autocamtide as a substrate) in extracts of mesangial cells that had been treated with 10 μM $CdCl_2$ for the times indicated. The results are representative of more than four experiments with both mouse and rat mesangial cells. In the experiment shown, autonomous activity was 17% of total activity at $t = 0$, rising to 37% at $t = 4$–6 h, as is typical of strong activation [39]. Significantly different from $t = 0$, $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$. A biphasic time course of phosphorylation and activity is apparent in panels A and C, respectively.

Table 2
Summary of flow cytometry findings

Conditions	Viable (%)	Apoptotic (%)	Late apoptotic/ necrotic (%)
Serum-free	83.0 ± 4.1	8.9 ± 3.9	7.4 ± 2.1
$CdCl_2$ (10 μM)	67.7 ± 10.1	23.7 ± 6.4	7.5 ± 3.7
$CdCl_2$ (10 μM) + KN93	74.9 ± 16.8	14.0 ± 10.6	9.5 ± 6.1
$CdCl_2$ (20 μM)	[a]18.8 ± 9.1	[b]56.2 ± 8.4	20.1 ± 13.1
$CdCl_2$ (20 μM) + KN93	[a]67.3 ± 9.2	[b]16.3 ± 4.9	15.0 ± 4.0

Values (mean ± S.D.) are compiled from three independent experiments of the type shown in Fig. 3. Cells are scored a viable (Annexin V −ve/PI −ve), apoptotic (Annexin V +ve/PI −ve), or late apoptotic/necrotic (Annexin V +ve/PI +ve). Values marked [a]differ significantly from one another, as do those marked [b]($P < 0.003$ in both cases). Cells staining Annexin V −ve/PI +ve were less than 2% in all experiments.

3. Results

Activation of CaMK involves autophosphorylation triggered by Ca^{2+}/calmodulin. Time course experiments at 10 μM Cd^{2+} showed a biphasic increase in phosphorylation of Thr_{286} of CaMK-II (phospho-CaMK-II) after 1 min and again after 4–6 h (Fig. 1A). We examined the concentration dependence of this activated form by Western blot at 6 h (Fig. 1B). Concentrations of 3–20 μM Cd^{2+} (a concentration range that activates MAPK signaling in mesangial cells [22,23]) markedly increased phospho-CaMK-II. Higher concentrations caused a loss of signal. Concentrations of 10 μM and higher also caused increased phosphorylation of Erk, as reported before [22], but in contrast to phospho-CaMK, phospho-Erk continued to increase even at 40 μM Cd^{2+} (Fig. 1B). The time course of phosphorylation of Erk was also biphasic, consistent with previous observations [22], with maxima lagging behind those of phospho-CaMK-II. The autonomous CaMK-II activity (Fig. 1C) corresponded to the pattern of phosphorylation of Thr_{286}, with an increase of approximately 2-fold at 5 min that declined, began to rise again at 2 h, and reached 3-fold at 4–6 h. Total CaMK-II activity measured in the presence of excess Ca^{2+} and calmodulin added to the assay mixture was unchanged over 6 h of Cd^{2+} treatment.

Based on the time course in Fig. 1, we looked at the effect of CaMK-II inhibitors on CaMK-II phosphorylation at 4 h and on Erk phosphorylation at 1 h and 6 h of Cd^{2+} treatment. The inhibitor KN93 suppressed the Cd^{2+}-dependent phosphorylation of CaMK-II, but the inactive analog KN92 did not (Fig. 2A). Neither compound affected CaMK-II phosphorylation in the absence of Cd^{2+}. Three other CaMK-II inhibitors, KT5926, K-252a, and AIP also blocked activation by Cd^{2+} (Fig. 2B). The CaMK-II-specific peptide analogue, AIP, decreased CaMK-II phosphorylation by 40% in two separate experiments. The lower efficiency of inhibition by AIP is likely due to peptide availability and the need for cell permeabilization for uptake. CaMK-II has been reported to signal upstream of Erk in vascular smooth muscle, with c-Src and the EGF receptor mediating ras/raf/MEK-dependent Erk phosphorylation [37]. KN93, but not KN92, decreased Cd^{2+}-dependent Erk phosphorylation (Fig. 2C), indicating Erk may also be a downstream target in the CaMK-II pathway in mouse mesangial cells. However, KT5926, K252a, and AIP did not decrease phospho-Erk levels (Fig. 2B), suggesting additional non-specific effects of KN93 on Erk.

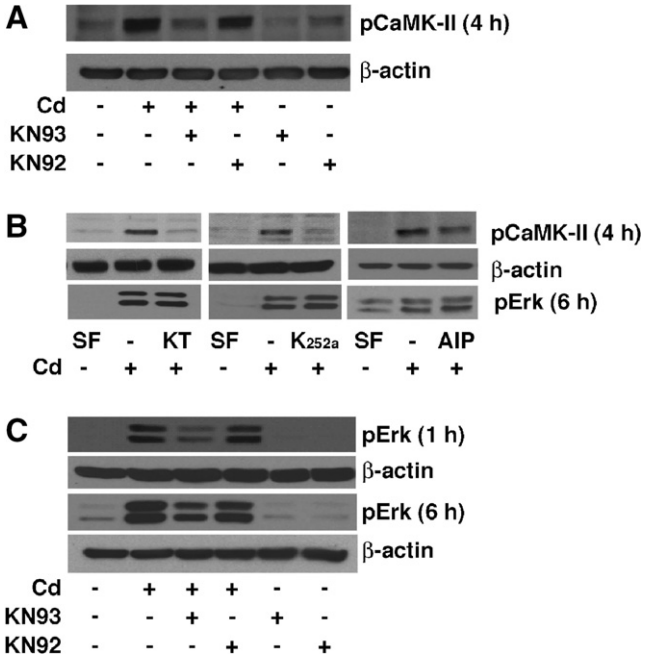

Fig. 2. Effect of CaMK-II inhibitors on Cd^{2+}-induced phosphorylation. (A) Western blots of phospho-CaMK-II and β-actin after treatment of cells in serum-free conditions for 4 h with or without 10 μM $CdCl_2$ in the presence or absence of KN93 or its inactive analog, KN92. (B) Western blots of phospho-CaMK-II, phospho-Erk, and β-actin after cells are held in serum-free (SF) conditions for 4 h or 6 h, or treated with 10 μM $CdCl_2$ (Cd) or $CdCl_2$ with the inhibitors KT5926 (KT), K-252a or AIP. (C) Western blots of phospho-Erk and the corresponding β-actin signal immediately below, after 1 h or 6 h treatment with 10 μM $CdCl_2$, KN93, or KN92, as indicated. All blots are representative of at least three independent experiments except AIP (tested twice).

Because CaMK-II has been implicated in regulating apoptosis, we determined the effect of Cd^{2+} and of CaMK-II inhibition on cell viability. For a 6 h exposure to 15 μM Cd^{2+}, this assay showed a decrease to 36 ± 11 % of the viability of serum-free cells alone (28.7% compared to quiescent cells before transfer to serum-free medium; Table 1). Inclusion of the CaMK-II inhibitor KN93 during Cd^{2+} treatment partially restored viability to 56 ± 7% of the serum-free value ($n = 6$, $P < 0.05$ vs. Cd^{2+} alone), whereas the Erk inhibitor PD98095 was without any protective effect, indicating that CaMK-II partially mediates Cd^{2+}-dependent cell death, and does so in an Erk-independent manner. K525a and AIP, which do not affect Erk phosphorylation, were also both protective of viability in the MTT assay (data not shown). Flow cytometry (Fig. 3) indicated that Cd^{2+} is killing the cells by both apoptotic (Annexin V +ve/PI −ve) and late apoptotic/necrotic (Annexin V +ve/PI +ve) pathways (see Section 4). KN93 and KT5926 protected cell viability, and did so markedly following a 6 h exposure to 20 μM Cd^{2+}. This was in large part due to a decrease in apoptosis (e.g., from 56 ± 8% of cells in 20 μM Cd^{2+} to 16 ± 5% when KN93 was included; Table 2).

4. Discussion

CaMK-II is the major CaMK in mesangial [26,39] and other smooth muscle [37,40] cells. It is activated upon binding of

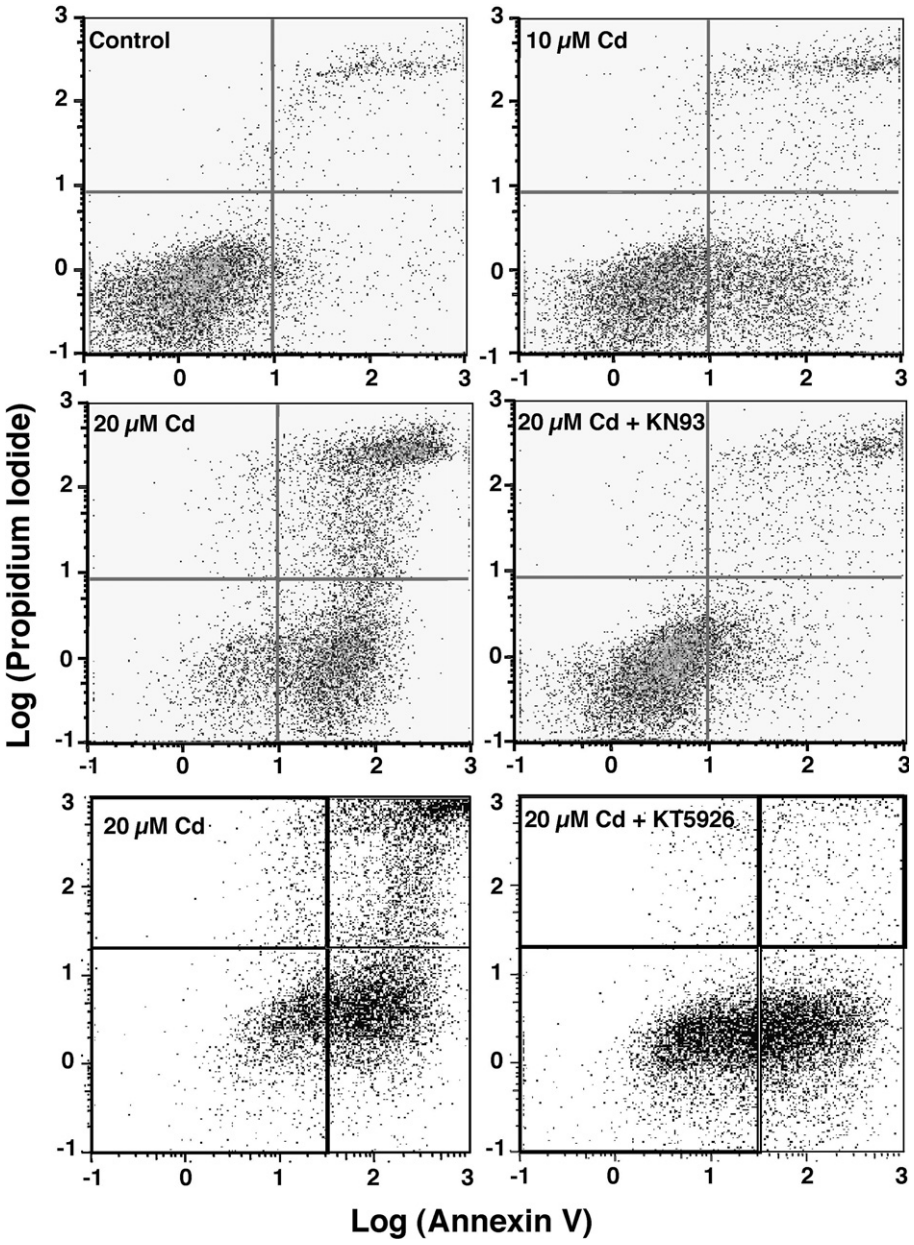

Fig. 3. Flow cytometric analysis of cell death. Cells were stained with Annexin V-FITC and propidium iodide as described in Section 2 and subjected to flow cytometry. Relative fluorescent intensity of the labels are indicated on the axes. Cells in the lower left quadrant of each panel are considered negative for both label, i.e., they are viable. Cells in the lower right quadrant are apoptotic. In these experiments, control cells are compared with cells treated for 6 h with 10 μM $CdCl_2$, 20 μM $CdCl_2$, or 20 μM $CdCl_2$ with KN93 or KT5926. Note that gating and correction for autofluorescence (effectively setting the axes for quadrant division) is optimized within each experiment, and each pair of horizontal images is matched from the same experiment. Results of multiple analyses are compiled in Table 2.

Ca^{2+}-containing calmodulin, undergoes autophosphorylation, and then takes on autonomous activity independent of calmodulin binding [41]. By virtue of its similar ionic radius to Ca^{2+}, Cd^{2+} can bind to calmodulin in vitro with a K_d of 4.5 μM and activate it to bind phosphodiesterase [29,42]. KN93 acts at the calmodulin binding site of CaMK to prevent binding, and suppression of Cd-dependent phosphorylation by KN93 indicates that Cd^{2+} is activating calmodulin binding in vivo. This is further confirmed with inhibitors KT5926, K252b, and AIP that act through different mechanisms (see Section 2), and a lack of effect of the analogue KN92.

We previously reported that Cd^{2+} did not increase total CaMK-II activity in rat mesangial cells at 1 h of treatment in serum-free medium, and at concentrations of 5 μM and above actually inhibited autonomous activity measured against autocamtide-2 peptide as substrate [30]. The lack of activation at 1 h is consistent with the present study. However, we show here that at both earlier and later times Cd^{2+} does activate CaMK-II. The mechanism of this biphasic effect is unknown. As Cd^{2+} enters mesangial cells by diffusion, linearly over a prolonged time period [43], the early activation of CaMK-II at 1–5 min may be due to mobilization of Ca^{2+}.

Cadmium was reported to mobilize cytosolic Ca^{2+} by binding to an orphan receptor on the cell surface [44]. However, we were unable to demonstrate a Ca^{2+} signal in response to Cd^{2+} in mesangial cells [45], and although this does not rule out Ca^{2+} acting locally or at privileged sites, it leaves the mechanism of the first phase of activation an open question. The second phase of activation may occur when intracellular Cd^{2+} levels are sufficient to activate calmodulin directly.

The prevention of CaMK-II Thr_{286} phosphorylation by the four CaMK inhibitors is good evidence of a specific effect of Cd^{2+} acting through CaMK-II. Activation of Erk by CaMK-II has been described in vascular smooth muscle cells, through a pathway involving EGF receptor downstream of CaMK-II [37]. However, in the present study, KT5926, K252a, and AIP had no effect on Erk, suggesting CaMK-II is not involved in Cd^{2+}-mediated Erk activation. The mechanism of suppression of Erk activation by KN93 is unknown, but is not due to a non-specific inhibitory effect of KN93 on the ras/raf/MEK/Erk arm of the signal, because KN93 had no effect on phorbol ester-induced Erk phosphorylation (data not shown).

As apoptotic cells may take up PI in late apoptosis, the flow cytometric method used here cannot distinguish late apoptosis from necrosis. However, the increase in the Annexin V +ve/PI +ve population with time and increasing Cd^{2+} concentration (data not shown, but compare 10 μM and 20 μM Cd in Fig. 3) suggests that the early effect of Cd^{2+} is primarily apoptotic in mesangial cells under serum-free conditions. It is noteworthy that inhibition of CaMK-II, but not of Erk, effectively suppresses apoptosis and increases viability of Cd^{2+}-treated cells in general, suggesting an apoptotic effect of CaMK-II independent of any role it may have in Erk signaling. Inhibition of phosphatases appears to lead to apoptosis through sustained CaMK-II activity [31,46] and CaMK inhibitors protected hepatocytes from DNA fragmentation after phosphatase inhibition [31]. The related kinases, CaMK-IV and CaMK-like kinase are both cleaved by caspase-3 to facilitate neuronal apoptosis [32,33]. However, CaMK is not consistently pro-apoptotic. Thus, CaMK-IV inhibits apoptosis in neurons deprived of K^+ [34] and CaMK-II maintains c-FLIP phosphorylation and renders Fas-sensitive glioma cells resistant to apoptosis [35].

In summary, we have demonstrated activation of CaMK-II by Cd^{2+} in intact mesangial cells, and implicated CaMK-II in Cd^{2+}-dependent apoptosis.

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